RHAMM, a receptor for hyaluronan-mediated motility, compensates for CD44 in inflamed CD44-knockout mice: A different interpretation of redundancy

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We report here that joint inflammation in collagen-induced arthritis is more aggravated in CD44-knockout mice than in WT mice, and we provide evidence for molecular redundancy as a causal factor. Furthermore, we show that under the inflammatory cascade, RHAMM (receptor for hyaluronan-mediated motility), a hyaluronan receptor distinct from CD44, compensates for the loss of CD44 in binding hyaluronic acid, supporting cell migration, up-regulating genes involved with inflammation (as assessed by microarrays containing 13,000 cDNA clones), and exacerbating collagen-induced arthritis. Interestingly, we further found that the compensation for loss of the CD44 gene does not occur because of enhanced expression of the redundant gene (RHAMM), but rather because the loss of CD44 allows increased accumulation of the hyaluronic acid substrate, with which both CD44 and RHAMM engage, thus enabling augmented signaling through RHAMM. This model enlightens several aspects of molecular redundancy, which is widely discussed in many scientific circles, but the processes are still ill defined.

The prevalent concept of molecular redundancy refers to the ability of a single receptor, enzyme, or DNA recognition element to interact with multiple ligands, substrates, or transcription factors, and vice versa. Thus, gene disruption is compensated by the redundant protein(s) and the relevant biological function is preserved (1–4). This important phenomenon is widely discussed in different scientific circles, but is far from understood at the molecular level. Analysis of exacerbated joint inflammation in collagen-induced arthritis (CIA) of CD44-deficient mice enlightens some aspects of the redundancy process.

The CIA model is, like human rheumatoid arthritis, characterized by fibrin deposition, synovial cell hyperplasia, mononuclear cell infiltrates, periosteal bone formation, and pannus formation (5) and depends highly on adhesion protein activity (6), including CD44 (7). Therefore, it is widely used as a model for the human disease.

Alternative splicing and differential glycosylation and attachment of glycosaminoglican chains generate multiple structural and functional versions of CD44. Proinflammatory activities such as cell–cell and cell–matrix interactions and support of cell migration are part of the CD44 functional inventory (reviewed in refs. 7–9). Hyaluronic acid (HA) (10) is the principal ligand of CD44 that mediates extravasation of $CD44^{+/+}$ white cells (11) and is expressed by activated endothelial cells of small blood vessels (12).

CD44 is a member of the link module superfamily HA-binding proteins (HABPs) or hyaladherins (13). Receptor for hyaluronan-mediated motility (RHAMM) is also a hyaladherin but lacks a link module. RHAMM is expressed on the cell surface (where it is designated CD168) and in the cytoplasm, as well as in the cytoskeleton and nucleus. Like CD44, RHAMM is subject to alternative splicing (14–17), particularly during tissue repair or after neoplastic conversion (17). After its interaction with HA, this receptor delivers signals for cell migration and proliferation in normal and malignant cells (15, 17–19).

Disruption of CD44 function by anti-CD44 mAbs (20–24) or hyaluronidase (24) reduces the pathology of experimental inflammatory diseases such as CIA (20, 21) and diabetes in NOD mice (24). CIA is, therefore, a CD44-dependent disease, and injection of type II collagen into CD44-deficient mice could reasonably be predicted to fail to induce joint inflammation. Here, we report that CIA is enhanced in CD44-deficient mice. We found that in the absence of CD44 a different hyaladherin, RHAMM, supports the inflammatory cascade, but even more efficiently than CD44. Exposure of the process underlying this event may allow a greater comprehension of molecular redundancy. Its mechanism is, perhaps, somewhat different and more complicated than that suggested by some of the accepted views.

Materials and Methods

Antibodies and Enzymes. The following antibodies were used in this study: rat anti-mouse CD44 constant region (IgG2b) mAb, obtained from hybridoma IM7.8.1 (ATCC, TIB-235) (25); rat anti-mouse CD44 constant region (IgG2b) mAb, obtained from hybridoma KM81 (ATCC, TIB-241) (26); rat anti-mouse cell surface Ig idiotype (IgG2b) mAb, obtained from hybridoma 4D2 (provided by J. Haimovich, Tel Aviv University, Tel Aviv) (27) and used as isotype-matched control; a migration blocking mouse anti-RHAMM mAb (IgG1) obtained from hybridoma 3T3.8 (a gift from L. Pilarski, University of Alberta, Edmonton, AB, Canada) (28) and IgG1 isotype-matched control Ig (Southern Biotechnology Associates); and rabbit anti-mouse RHAMM IgG polyclonal antibody (R218) and rabbit IgG (preimmune serum) (16). The enzymes heparinase (H-2519) and testicular hyaluronidase (H-3757) were obtained from Sigma. HA was derived from bovine trachea (Sigma, HO902). An N-terminal truncated form of a soluble recombinant RHAMM (rRHAMM) fused to GST was produced as described (18).

Mice and Induction of CIA. CD44-deficient mice (29) were backcrossed for seven generations onto a DBA/1 background ($H\text{-}2^q$) and typed by PCR, using tail biopsy-derived DNA. Genomic PCR was performed by using primers and conditions as described (29). CIA was generated in $CD44^{-/-}$, $CD44^{+/-}$, and $CD44^{+/+}$ male littermates, 10–14 weeks of age, by immunization with one or two doses (given 3 weeks apart) of 200 μg of type II collagen derived from bovine articular cartilage as described (30) and emulsified in

Abbreviations: RHAMM, receptor for hyaluronan-mediated motility; rRHAMM, recombinant RHAMM; CIA, collagen-induced arthritis; HA, hyaluronic acid; HABP, HA binding protein.

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complete Freund's adjuvant (21). The thickness of each affected hind paw was measured with microcalipers, and disease activity was expressed as paw width in mm (21). To evaluate the anti-constant CD44 mAb (anti-pan CD44 mAb) or anti-RHAMM mAb effect on CIA, each mouse was i.p. administered 100 μl of PBS or 150 μg of antibody (or isotype-matched control antibody) in 100 μl of PBS on the day of disease onset and then every other day for 8–14 days. To evaluate the effect of hyaluronidase on CIA, hyaluronidase (20 units per mouse), heparinase (equivalent specifically active control, 10 units per mouse), or PBS (100 μl) was injected i.p. into each mouse on the day of first injection of collagen (day 0) and then every other day for 5 weeks. To evaluate the effect of soluble rHAMM on CIA, GST-rHAMM (1 mg/kg per mouse) or GST (1 mg/kg per mouse) was injected i.p. into each mouse on the day of the first injection of collagen and then twice a week for 5 weeks.

Transfer Experiment. CD44$^{-/-}$ and CD44$^{+/+}$ male donor DBA/1 mice were killed, the spleens were removed, and suspensions of spleen leukocytes were washed twice in PBS. A quantity of 25×10^6 leukocytes of each cell phenotype was injected i.v. into each irradiated (750 cGy) male recipient mouse. The mice were immunized with one 200-μg dose of collagen 24 h after leukocyte transfer.

Flow Cytometry. For flow cytometry, 10^5 spleen leukocytes or joint-infiltrating cells were incubated with antibody for 45 min on ice. After extensive washing, the cells were incubated with FITC-conjugated secondary anti-Ig antibody (Jackson ImmunoResearch) for 30 min on ice. The cells were then washed and analyzed by flow cytometry (Becton Dickinson). Flow cytometry data were collected for viable cells according to side scatter and forward scatter. Joint-infiltrating cells were isolated from the synovial membrane of arthritic joints after shaking the membrane in RPMI medium 1640, containing 0.1 mg/ml DNase (Worthington) and 1 mg/ml collagenase (Worthington) for 30 min at 37°C. After centrifugation, the joint-infiltrating cells were washed and analyzed by flow cytometry.

Histology and Histochemistry. Arthritic paws were removed postmortem and fixed in 10% (wt/vol) buffered formalin. After decalcification in buffered formalin containing EDTA (5.5% wt/vol), the paws were embedded in paraffin, sectioned, and stained with hematoxylin and eosin or safranin-o for microscopic evaluation.

For detection of HA, tissue sections were prepared as described above were assessed for the presence of HA, using HABP, as described in ref. 24. Stained sections were digitally photographed at ×40 magnification at identical exposure times and saved as TIF files. The images were then imported into PHOTOSHOP (Adobe Systems, San Jose, CA), reduced by 50%, and converted to gray-scale with a threshold level of 130. The histogram function was used to determine the pixel density per unit image. The average pixel density for tissue sections that had been exposed to secondary reagent only were subtracted from the average pixel density for experimental sections in which HABP had been included to detect HA. The pixel density of tissue sections obtained from animals that had received injections of hyaluronidase served as controls for the specificity of the HABP probe.

cDNA Microarray. cDNA microarrays containing ≈13,000 mouse cDNA clones on three physically different slides were used in this study as described (31, 32). cDNA clones were obtained commercially from Research Genetics (Huntsville, AL) (I.M.A.G.E. Consortium), Incyte Genomics (Palo Alto, CA), and internal sources. Normalized data were used to compare differences in gene expression between T cells from CD44 knockout mice and WT mice.

Migration Assay. Transwell migration assays in Boyden chambers were performed as described (33), using a polycarbonate filter coated with HA (10 μg per filter). Spleen leukocytes were suspended, washed, and resuspended in DMEM containing 0.1% BSA and then introduced (4×10^5 cells) into the upper compartment of a Boyden chamber with 20 μg of antibodies or without antibodies. Cells that traversed the HA layer and attached to the lower surface of the filter were stained with Diff Quick (American Scientific Products, McGraw Park, IL) and counted. The results represent three independent experiments, each performed in duplicate.

Western Blot Analysis. Spleen leukocytes were lysed in Nonidet P-40 and Western-blotted, as described (34), using 1 μg of anti-RHAMM polyclonal antibody (R218) per ml to detect the RHAMM isoforms.

Statistical Analysis. Data were analyzed by using microcomputer programs for one-way ANOVA, followed by Student's t test for unpaired values. $P < 0.05$ was considered significant. The results are

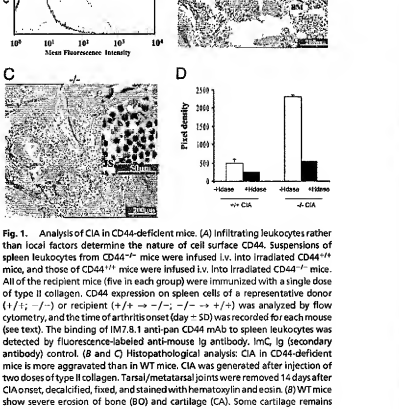

Fig. 1. Analysis of CIA in CD44-deficient mice. (*A*) Infiltrating leukocytes rather than local factors determine the nature of cell surface CD44. Suspensions of spleen leukocytes from CD44$^{-/-}$ mice were infused i.v. into irradiated CD44$^{+/+}$ mice, and those of CD44$^{+/+}$ mice were infused i.v. into irradiated CD44$^{-/-}$ mice. All of the recipient mice (five in each group) were immunized with a single dose of type II collagen. CD44 expression on spleen cells of a representative donor (+/+; −/−) or recipient (+/+ → −/−; −/− → +/+) was analyzed by flow cytometry, and the time of arthritis onset (day ± SD) was recorded for each mouse (see text). The binding of IM7.8.1 anti-pan CD44 mAb to spleen leukocytes was detected by fluorescence-labeled anti-mouse Ig antibody. ImC, Ig (secondary antibody) control. (*B* and *C*) Histopathological analysis: CIA in CD44-deficient mice is more aggravated than in WT mice. CIA was generated after injection of two doses of type II collagen. Tarsal/metatarsal joints were removed 14 days after CIA onset, decalcified, fixed, and stained with hematoxylin and eosin. (*B*) WT mice show severe erosion of bone (BO) and cartilage (CA). Some cartilage remains intact, the joint space (JS) is still evident, and the joint architecture is partially maintained. (*C*) CD44-deficient mice show severe erosion of bone and complete erosion of cartilage. The entire joint architecture (including joint space) has been obliterated. (*Inset*) Infiltration of a large number of polymorphonuclear leukocytes into the joint space of CD44-deficient mice. The joint was removed at disease onset, and the section was stained with safranin-o after fixation and decalcification. BM, bone marrow. (*D*) Histochemical photoscanning analysis: Enhanced accumulation of HA in joint tissues of arthritic CD44$^{-/-}$ mice. Tarsal/metatarsal joints were removed 14 days after the onset of CIA in arthritic WT (+/+ CIA) and arthritic CD44-deficient (−/− CIA) mice, subjected (+Hdase) or not subjected (−Hdase) to hyaluronidase treatment, as described. HA accumulation in tissue was identified by a biotinylated HABP and avidin-biotin peroxidase detection system. The intensity of HA accumulation in the tissue was analyzed by photoscanning, as described in *Materials and Methods*.

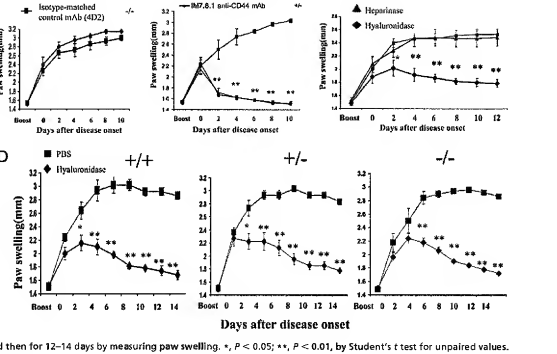

Fig. 2. The compensating molecule in CD44-deficient mice recognizes HA. (*A* and *B*) CIA in CD44-deficient mice is refractory to treatment with anti-CD44 mAb. CD44$^{-/-}$ (*A*) and CD44$^{+/+}$ and CD44$^{+/-}$ (*B*) DBA/1 mice (five in each group), showing symptoms of arthritis after two injections of type II collagen, were treated upon disease onset and then every other day for 10 days with 150 μg of IM7.8.1 anti-CD44 mAb or isotype-matched control mAb (see symbols). Arthritis development was monitored for 10 days by measuring paw swelling. (*C* and *D*) CIA in CD44-deficient mice is sensitive to enzymatic treatment with hyaluronidase. WT (*C* and *D Left*), heterozygous (*D Center*), and CD44-deficient (*D Right*) DBA/1 mice (five in each group), in which CIA was induced after two doses of type II collagen, were treated at the initiation phase of the disease (day 0) and then every other day for 5 weeks with 20 units of hyaluronidase (◆), 10 units (equivalent specific activity) of heparinase (▲), or PBS (■). Arthritis development was monitored from disease onset and then for 12–14 days by measuring paw swelling. *, $P < 0.05$; **, $P < 0.01$, by Student's *t* test for unpaired values. The results are expressed as the mean ± SE.

expressed as the mean ± SEM. Each experiment was repeated at least twice and in most cases three times, with all of the experiments showing similar results.

Results

Characterization of CIA in CD44-Deficient DBA/1 Mice. CD44-deficient mice (29) provided by Tak Mak (Ontario Cancer Institute, Toronto) were back-crossed for seven generations onto a DBA/1 background to establish a CD44-deficient mouse line susceptible to CIA. It has been reported (29, 35, 36) that CD44 knockout mice display an almost normal phenotype, with only a few exceptions, e.g., impaired lymphocyte homing to the lymph nodes and thymus (35). PCR and flow cytometry analyses confirmed the loss of CD44 in knockout mice (CD44$^{-/-}$), CD44 expression in WT mice (CD44$^{+/+}$), and intermediate CD44 expression in heterozygous mice (CD44$^{+/-}$) (Fig. 5, which is published as supporting information on the PNAS web site). WT mice (five animals) did not develop arthritis, even 40 days after injection of collagen, unless challenged with a second dose, which induced arthritis within 5 (±2) days (retarded CIA). Surprisingly, CD44-deficient mice (five animals) developed arthritis 25 (±1) days after injection of a single dose (200 μg) of collagen. Hence, CD44-deficient DBA/1 mice are more susceptible to CIA than WT DBA/1 mice.

To determine the role of the environment vs. that of infiltrating white cells in mediating the enhanced disease, splenocytes were transferred from WT mice into irradiated CD44-deficient mice, and vice versa. All of the mice received an injection of type II collagen 24 h after cell transfer. The genotype (CD44$^{-/-}$ or CD44$^{+/+}$) of the transferred splenocytes, rather than local factors of the recipients, dictates whether the recipient spleen cell phenotype is CD44$^{-/-}$ or CD44$^{+/+}$ (Fig. 1*A*) and whether CIA in the recipient mice (five per group) is accelerated [i.e., CIA develops 26 (±2) days after transfer of CD44$^{-/-}$ cells into CD44$^{+/+}$ mice and a single injection of collagen] or retarded [i.e., CIA develops 43 (±2) days after transfer of CD44$^{+/+}$ cells into CD44$^{-/-}$ mice and two injections of collagen].

In all subsequent experiments, CIA was induced after two injections of collagen 3 weeks apart. Histopathology of joint tissue removed 14 days after disease onset showed that CIA in WT mice (Fig. 1*B*) was less severe than in CD44-deficient mice (Fig. 1*C*) and that more extensive chondrolysis was observed in CD44$^{-/-}$ joints. However, polymorphonuclear leukocytes were the predominant inflammatory cell at disease onset (Fig. 1*C Inset* and results not shown) in both cases. Interestingly, histochemical examination of HA, using HABP as a specific probe for this glycosaminoglycan, showed enhanced accumulation of HA in the joint tissue of CD44-deficient mice (Fig. 1*D*), when compared with WT mice, as indicated by histochemical photoscanning (Fig. 1*D*). The HA accumulation was markedly reduced after hyaluronidase injection. Because cell surface CD44 is required for binding, internalizing, and intracellular digestion of HA (37), excess HA, in the absence of CD44 (i.e., in CD44-deficient mice), may tend to accumulate, intensifying the inflammatory cascade.

This histopathologic observation was confirmed by microarray analysis of RNazol-extracted total RNA derived from splenic T cells, isolated by anti-CD3 mAb-coated beads and magnetic cell sorting. The T cells were obtained from arthritic or nonarthritic WT and CD44$^{-/-}$ mice at disease onset. RNA hybridization to microarrays containing ≈13,000 mouse cDNA clones was performed and those with at least 2-fold changes in expression were considered differentially expressed transcripts. The number of transcripts that changed upon induction of arthritis was much greater in CD44$^{-/-}$ mice than in WT mice (170 vs. 47). When arthritic WT and CD44$^{-/-}$ mice were compared, 53 changes in transcript expression were observed (Table 1, which is published as supporting information on the PNAS web site), of which at least 18 were associated with up-regulation of genes involved directly or indirectly in inflammation (Table 2, which is published as supporting information on the PNAS web site). These findings support the concept that CD44 deficiency synergizes with a process enhancing arthritic activity, thereby increasing the number of up-regulated inflammation-involved transcripts (including migration-supporting genes). This process results in perpetuation of the disease and increased joint

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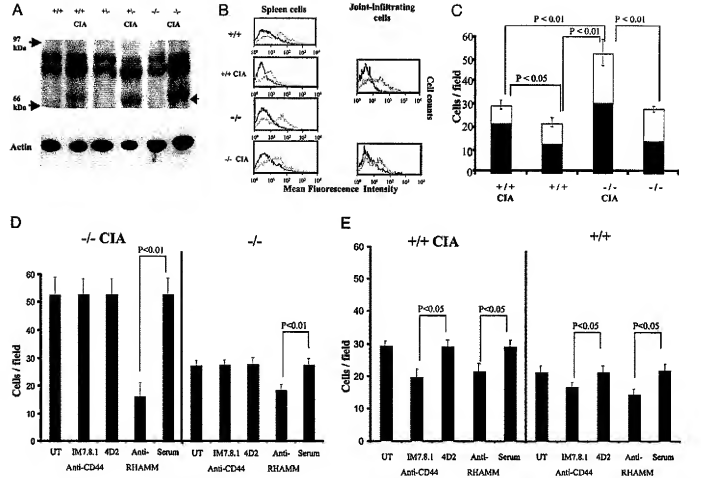

Fig. 3. Expression and migratory function of RHAMM in spleen leukocytes of arthritic and nonarthritic CD44-deficient and WT mice. (*A*) Western blot analysis. Extracts of spleen leukocytes from arthritic (CIA) and nonarthritic CD44$^{+/+}$, CD44$^{+/-}$, and CD44$^{-/-}$ DBA/1 mice, treated twice with collagen, were analyzed by Western blot using polyclonal anti-RHAMM antibody. (*B*) Flow cytometry analysis. Cell surface RHAMM was assessed on spleen leukocytes (*Left*) from arthritic (CIA) or nonarthritic CD44$^{+/+}$ and CD44$^{+/-}$ DBA/1 mice or on joint-infiltrating cells derived from arthritic CD44$^{+/+}$ and CD44$^{-/-}$ mice (*Right*) by measuring the binding of polyclonal anti-RHAMM antibody to the cells. The binding of anti-RHAMM antibody was identified with anti-rabbit Ig conjugated to fluorescein. The black line in each histogram shows the nonspecific binding of second antibody alone. (*C*) Enhanced transwell migration of CD44-deficient spleen leukocytes. Spleen leukocytes derived from arthritic (CIA +/+ and CIA −/−) and nonarthritic (+/+ and −/−), WT (+/+), and CD44-deficient (−/−) DBA/1 mice were subjected to the transwell migration assay. The spleen leukocytes were analyzed for their ability to cross filters, noncoated (filled bars) or coated (empty + filled bars) with HA, toward conditioned fibroblast medium that served as chemoattractant. The number of cells that traversed the filter are shown on the *y* axis. (*D* and *E*) Inhibition of transwell migration of spleen leukocytes from arthritic CD44-deficient mice by anti-RHAMM antibody. Transwell migration (across HA-coated filters) of arthritic (−/− CIA; +/+ CIA) and nonarthritic (−/−; +/+) spleen leukocytes from CD44-deficient (*D*) and WT (*E*) mice was analyzed as described in *C*. The assay was performed in the presence of medium (UT, untreated), anti-RHAMM polyclonal antibody (Anti-RHAMM), preimmune rabbit serum (serum), and IM7.8.1 anti-CD44 mAb and isotype-matched control mAb (4D2). Statistical analysis was done by Student's *t* test for unpaired values. The results are expressed as the mean ± SE.

deterioration. Notably, the microarray analysis of CD44$^{-/-}$ cDNA detected increased expression of jun and fos, as well as increased expression of proinflammatory genes regulated by AP-1, which is formed from jun and fos dimers. Therefore, the processes involved in enhancing joint destruction in the absence of CD44 are associated with altered HA metabolism and up-regulation of inflammatory mediators, particularly those linked to cell migration and AP-1 formation.

The Compensating Molecule in CD44-Deficient Mice Recognizes HA. As expected, injection of anti-pan CD44 mAb into CD44-deficient mice did not reduce CIA, as their footpads swelled to the same extent as in animals receiving isotype-matched control mAb (4D2) (Fig. 2*A*). In contrast, and as expected, CIA was reduced after injection of anti-pan CD44 mAb in WT and heterozygous mice compared with that in controls receiving isotype-matched mAb (4D2) (Fig. 2*B*). These findings suggest that CIA in CD44-deficient mice is mediated by a molecule that compensates for CD44 but maintains at least some of its functions (e.g., support of neutrophil extravasation), a phenomenon known as redundancy.

Injection of hyaluronidase, but not PBS (Fig. 2 *C* and *D*) or heparinase (Fig. 2*C*), reduced CIA in WT, heterozygous, and CD44-deficient mice to the same extent. These findings imply that cleavage of HA [and perhaps also chondroitin sulfate (CS), which can be cross-cleaved with HA] inhibited pathological manifestations of CIA in the presence or absence of CD44 and suggest that the compensating molecule(s) in CD44-deficient mice might be another HA (and/or CS) receptor. This possibility is supported by our finding that splenocytes from both CD44-deficient and WT mice bound to immobilized HA to the same extent, regardless of whether they were derived from arthritic or nonarthritic mice (Fig. 6, which is published as supporting information on the PNAS web site). A survey of the literature for HABPs suggested RHAMM as a possible candidate for a CD44-compensating molecule, because this hyaladherin binds to HA (but not to CS) (38–40), mediates leukocyte trafficking (17), and promotes expression of both jun and

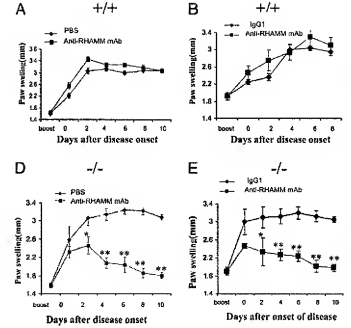

Fig. 4. CIA in CD44-deficient mice is sensitive to treatment with anti-RHAMM mAb or soluble rRHAMM. $CD44^{+/+}$ (*A–C*) and $CD44^{-/-}$ (*D–F*) DBA/1 mice, treated twice with type II collagen to induce CIA, were administered 150 μg of anti-RHAMM mAb or PBS (*A* and *D*, eight mice in each group), 150 μg of anti-RHAMM mAb or isotype-matched IgG1 (*B* and *E*, five mice in each group), using the protocol described in Fig. 3, or 1 mg/kg soluble mouse GST-rRHAMM or GST (*C* and *F*, eight mice in each group), administered the day of the first collagen injection and then twice a week for 5 weeks. Arthritis development was monitored for 8–10 days by measuring paw swelling. *, $P < 0.05$; **, $P < 0.01$, by Student's *t* test for unpaired values. The results are expressed as the mean ± SE.

fos in response to HA (19). Therefore, if CIA in CD44-knockout mice is RHAMM-dependent, this phenomenon should be associated with HA rather than with CS interaction.

RHAMM Compensates for CD44 in Arthritic CD44-Deficient Mice. Western blot analysis of splenocyte extracts with anti-RHAMM antibody revealed that in DBA/1 mice CIA induced the expression of a RHAMM isoform (70 kDa) in both CD44-deficient and WT mice. The 70-kDa band is specific because it is absent from $RHAMM^{-/-}$ cell extracts. The doublet bands are not specific because they are detected in $RHAMM^{-/-}$ cell extracts, as well as in cell extracts lacking RHAMM because of small interfering RNA treatment (results not shown). The same 70-kDa RHAMM protein was detected after scratch wounding *in vitro* and excisional wounding *in vivo* (41). The level of the 70-kDa RHAMM protein expression detected in splenocyte extracts of arthritic CD44-deficient, WT, and heterozygous mice was similar (Fig. 3*A*), as indicated by the densitometric RHAMM signal/actin signal ratio (data not shown). Immunostaining with anti-RHAMM polyclonal antibody revealed that RHAMM was not up-regulated on the cell surface (Fig. 3*B Left*) and the intracellular components (data not shown) of spleen leukocytes from WT and CD44-deficient mice, with or without CIA. RHAMM was also not up-regulated on joint-infiltrating cells of arthritic CD44-deficient mice when compared with that of arthritic WT mice (Fig. 3*B Right*). These results were further confirmed by microarray analysis, showing no difference in RHAMM gene expression between WT and CD44-deficient mice (results not shown). Hence, RHAMM protein expression is not up-regulated in CD44-deficient mice, and CIA, but not the presence or absence of CD44, influences the isoform expression of RHAMM.

In transwell assays, spleen leukocytes from arthritic CD44-deficient mice migrated across noncoated filters and filters coated with HA (Fig. 3*C*) or fibronectin (data not shown) at higher rates than spleen leukocytes from nonarthritic CD44-deficient mice or arthritic and nonarthritic WT mice. Notably, HA-coated filters augmented transwell migration of all splenocyte types. However, even in the absence of HA, spleen cells from arthritic $CD44^{-/-}$ mice displayed higher migratory activity than those obtained from WT mice. This observation is consistent with the microarray analyses (Table 2), which showed enhanced expression of genes associated with inflammation (including motility receptors) in arthritic $CD44^{-/-}$ mice. We had shown CD44-dependent but HA-independent transwell migration of hematopoietic stem cells (42).

Importantly, incubation of the arthritic and nonarthritic CD44-deficient spleen cells with anti-RHAMM antibody (but not with preimmune serum, IM7.8.1 anti-CD44 mAb, or isotype-matched 4D2 control mAb) reduced splenocyte migration across HA-coated filters (Fig. 3*D*). Assessment of splenocytes removed from arthritic and nonarthritic WT mice revealed a slight (but yet significant, $P < 0.05$) reduction in cell migration after incubation with IM7.8.1 anti-CD44 mAb or anti-RHAMM antibody when compared with incubation with the relevant controls (Fig. 3*E*). These results show that RHAMM supports splenocyte migration to a much greater extent when such spleen cells are deficient in CD44.

As RHAMM is active in $CD44^{-/-}$ splenocytes (Fig. 3), we next evaluated the *in vivo* effect of anti-RHAMM mAb prepared against embryonic murine fibroblasts or soluble GST-rRHAMM (previously shown to block ras-mediated *in vivo* cell functions) (18) on CIA in WT and CD44-deficient mice. Injection of the anti-RHAMM mAb (Fig. 4 *A* and *B*) or soluble GST-RHAMM (Fig. 4*C*) did not influence the course of CIA in WT mice, but markedly reduced CIA in similarly treated CD44-deficient mice (Fig. 4 *D–F*). Injection of isotype-matched IgG1 or GST did not influence CIA.

Discussion

Our findings imply that CIA in WT mice is CD44-dependent, whereas in CD44 knockout mice it is RHAMM-dependent, as determined by antibody blocking and soluble peptide competition studies. We suggest that in the WT mice CD44 and RHAMM coexist, but cell surface CD44 function is dominant. Cell surface CD44 may primarily influence the joint inflammatory cascade in WT mice by its ability to quantitatively compete with cell surface RHAMM for HA and/or its ability to regulate the potency of RHAMM-mediated signaling that may or may not be HA-dependent. In the absence of CD44, i.e., in CD44-deficient mice, the highly potent cell surface RHAMM can induce an even more aggressive response that at least in part is caused by its interaction with HA (19). In line with this view, greater HA accumulation was detected in the arthritic joints of CD44-deficient mice than in those of WT mice, because in the latter cell surface CD44 (but not RHAMM) could promote endocytosis of HA and its subsequent

delivery for lysosomal digestion (9). The excess HA in CD44$^{-/-}$ mice may contribute to prolonged signaling through RHAMM (17), which might lead to aggravation of the inflammatory disease. Thus, the compensation for loss of the CD44 gene does not occur because of enhanced expression of the redundant gene (RHAMM), but rather because the loss of CD44 allows enhanced accumulation of the HA substrate, with which both CD44 and RHAMM engage. Although RHAMM is not hyperexpressed in the absence of CD44, other proinflammatory genes, many of which have been linked to RHAMM and are regulated through RHAMM signaling, are activated in arthritic CD44$^{-/-}$ mice. For example, RHAMM is required for activation of signaling pathways through IL-1β and TNF-α, such as erk, and AP-1 (14, 19). RHAMM also binds to calmodulin (16), regulates connexin expression (43), and promotes formation of diacylglycerol in response to hyaluronan, linking this hyaladherin to phospholipase Cγ (44).

Although in many cases redundant proteins are structurally and functionally related (1–4), RHAMM and CD44 belong to discrete molecular families, although they share HA binding and promigratory functions. Contrary to prevailing views, the replacement of CD44 function by RHAMM in CD44-deficient mice is not associated with any change in RHAMM mRNA transcription or a specific RHAMM isoform, which is expressed in CIA of WT and CD44$^{-/-}$ mice (Fig. 3). Nevertheless, RHAMM appears to efficiently support cell motility and CIA development when free from CD44 constraint in CD44-deficient mice. In contrast, CD44 targeting (e.g., by anti-CD44 mAb) in adult WT mice is not sufficient to free RHAMM for support of CIA development. These observations suggest that the continuous and rigorous requirements for the replacement of CD44 during the embryogenesis of CD44 knockout mice lead to an increase, in a still unknown manner, in the potency of RHAMM. This change allows RHAMM to compensate for the lack of CD44 not only during embryogenesis, but also in adulthood. This may not be the case when CD44 is targeted by antibody, for a short period, in the adult animal. However, in the *in vitro* system, both anti-RHAMM and anti-CD44 antibodies slightly, but significantly, inhibit cell migration of WT splenocytes (Fig. 3). This finding suggests that RHAMM is partially potent under the limited *ex vivo* conditions, which likely expose the cells to growth factors that may require RHAMM function (17).

Acceleration of CIA resulting from loss of CD44 is similar to exacerbation of bleomycin-induced lung inflammation previously reported in CD44-deficient mice (45). In that study, it was concluded that the constrained lung inflammation observed in WT mice results from the involvement of CD44 in limiting inflammation by factors such as TGF-β. Our findings suggest an alternative explanation for the increased lung inflammation observed in the absence of CD44: replacement of the deleted CD44 gene by a potent redundant gene product that misregulates these CD44-mediated functions, thus leading to severe tissue damage.

Our results differ somewhat from those reported for CIA in CD44$^{-/-}$ mice by Stoop *et al.* (36). Although they found that CIA was less severe in CD44 knockout mice, the bulk of joint inflammatory reaction was in fact persistent in these animals. This fact implies that the difference between their results and ours is not a prominent one and also raises the possibility of a molecular redundancy in their model. Furthermore, in line with our findings (21), the effect of anti-CD44 mAb on CIA in their study (20) was clearly more substantial than the effect of CD44 gene deletion (36). The moderate discrepancy between the two models can be explained by disparate mouse inbreeding in the two laboratories ("genetic deviation"), different sources of type II collagen, and variations in disease induction protocols. These factors may generate slightly different diseases as indicated by distinct disease onsets. In addition, disease evaluation parameters were dissimilar. Intriguingly, mice genetically deleted of a CD44 variant (CD44v7) recovered completely from experimental colitis because this deletion results in apoptosis of inflammatory cells (46). These and our studies suggest that CD44 deletions will enable dissection of the roles of CD44 and other molecules in a variety of inflammatory processes.

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Correction

IMMUNOLOGY. For the article "RHAMM, a receptor for hyaluronan-mediated motility, compensates for CD44 in inflamed CD44-knockout mice: A different interpretation of redundancy," by Shlomo Nedvetzki, Erez Gonen, Nathalie Assayag, Reuven Reich, Richard O. Williams, Robin L. Thurmond, Jing-Feng Huang, Birgit A. Neudecker, Fu-Shang Wang, Eva A. Turley, and David Naor, which appeared in issue 52, December 28, 2004, of *Proc. Natl. Acad. Sci. USA* (**101,** 18081–18086; first published December 13, 2004; **10.1073/pnas.0407378102**), the name Fu-Shang Wang should have appeared as Fu-Sheng Wang. The online version has been corrected. The corrected author line appears below.

Shlomo Nedvetzki, Erez Gonen, Nathalie Assayag, Reuven Reich, Richard O. Williams, Robin L. Thurmond, Jing-Feng Huang, Birgit A. Neudecker, Fu-Sheng Wang, Eva A. Turley, and David Naor

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